A Droplet Microarray for Homogeneous Enzymatic Assay

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ABSTRACT

This work describes a droplet microarray for monitoring of enzymatic activity on a glass surface. These array are composed of features defined and separated by differential surface tension (surface tension array). A photolithographic method was used to create a series of circular hydrophilic spots delimited by a perfluorosilanated surface. The perfluorinated region had water contact angle on the order of 105°, whereas inside the spots this angle was < 75°. The difference in surface tension "holds" water within the hydrophilic spots thus resulting in droplet microarray. We show that each droplet can be used as a micro-reservoir for conducting of enzymatic reactions. As a proof of concept a proteolytic cleavage of a fluorogenic substrate was carried out within hundreds of confined reservoirs created on a glass slide. Using piezoelectric deposition of substrate and protease numerous reaction conditions could be tested in parallel. This format may be a valuable tool for high-throughput screening, and enzymatic profiling of novel proteins.

***Keywords*:** microarray, droplets, screening, proteases, fluorescence

1. INTRODUCTION

Microarrays offer a convenient platform for multiplex protein analysis. However, until recently protein array technology was limited to analysis of protein-protein interactions and did not provide information about an enzymatic activity. Yet a wide variety of therapeutic targets are enzymes and conventional high throughput screening (HTS) assays of drug libraries are often based on monitoring of enzymatic reaction.

Proteases, enzymes hydrolyzing a peptide bond, are the key regulators of cellular function and viability. They are involved in many important biological processes including protein maturation and degradation, cellular signaling, cell death and differentiation etc. Furthermore, proteases play a central role in diverse pathologies, such as inflammation, cancer, pathogen infections, and Alzheimer's disease, making these enzymes important therapeutic targets and disease biomarkers [1,2]. A number of high throughput techniques have been proposed for the determination of protease substrate specificity and inhibitor screening, though they do not generally provide a complete kinetic analysis of protease activity [3,4].

Few recent works described the application of a microarray slide format for enzymatic analysis [5-7]. All these approaches are heterogeneous in term that either an enzymatic substrate [5,6] or an enzyme [7] is covalently bound to the slide surface. The heterogenic nature of the assay results in more complicated enzymatic kinetics that can hardly been compared with conventional enzymatic reaction in solution. Moreover the binding of the reagent to the surface requires special coupling chemistry been applied for the substrate or enzyme.

We report here the assembly and evaluation of droplet microarrays that enable high throughput homogeneous enzymatic assay. This method can be used for protease characterization and inhibitor screening.

2. RESULTS

2.1 Device: Droplet Microarrays

To meet our aim of analyzing various conditions for proteolytic reactions simultaneously, we needed microarrays supports able to confine hundreds of reaction vessels on a small area. These microarrays are composed of features defined and separated on the glass slide by differential surface tension (surface tension arrays). Specifically, the photolithographic approach of Butler *et al*. [8] was used to create a series of hydrophilic spots bounded by a perfluorinated surface. The differences in surface energies between the spots and surrounding zones allow for droplets to be readily formed within a defined site. Microarrays of 300 to 3000 hydrophilic features (500μm in diameter) were used in protease assays (Figure 1).

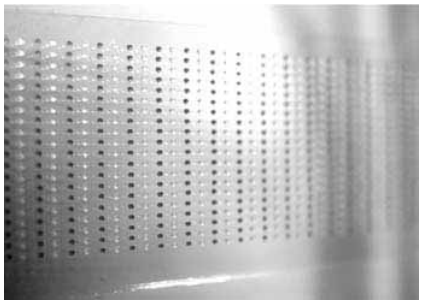

Figure 1: Droplet microarray on a glass slide. Reservoirs consist of 500 μm features defined and separated on a glass slide by differential surface tension. Microarrays of 300 to 3000 hydrophilic features can be built and typical droplet volume is 20 nL.

2.2 Experimental Design

The droplets arrayed on the surface of glass slides served as microreactors for proteolysis reactions. The robot used for dispensing reagents in the microarray allows precise targeting of each individual hydrophilic spot. Drop's patterns can easily be designed in order to test a variety of conditions in a single experiment.

The protease assay was carried out as follow: According to a plan of experiment, protease, fluorogenic substrate and protease inhibitor were mixed directly in droplets by using piezoelectric dispense system. We varied enzyme and substrate concentrations with appropriate no-enzyme and inhibitor controls, creating a series of droplet groups housing diverse reaction conditions. The progress of each individual reaction was followed by liberation of the fluorescence due to the substrate cleavage and was measured using a Genomic Solution array scanner. After quantification and data treatment the kinetic of proteolysis could be precisely monitored.

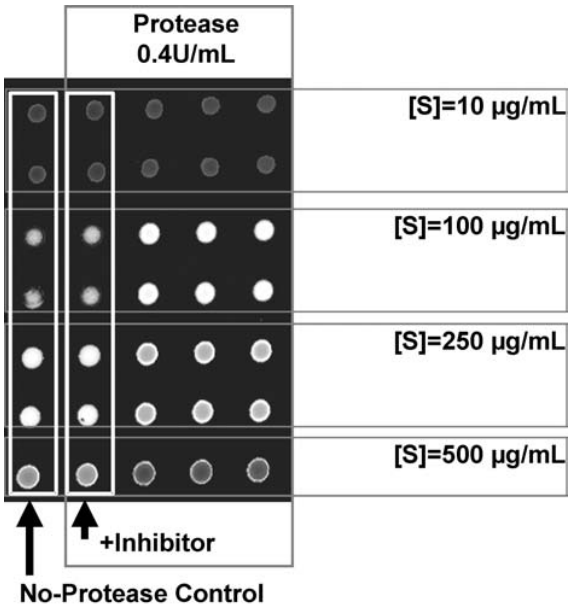

Figure 2: Typical image of the proteolytic reaction obtained with slide array scanner

2.3 Monitoring of proteolytic activity

In a proof of concept experiment we observed that proteolysis was occurring, using a no-protease control and an inhibitor control (Figure 2). Kinetics of proteolysis and the effect of inhibitor could be determined in parallel for each individual droplet. After data collection and treatment, the mean values and standard deviations against time could be plotted for each group of droplet housing the same reaction. The reaction proved to be extremely slow at room temperature [from 340 to 1220 min] but proceeded with an acceptable rate (over a few hours) at 37°C [0 to 340 min and 1220 to 1645 min].

Application of microarray technology allowed rapid optimization of the experimental conditions. Indeed, within a single experiment, we tested forty different conditions (eight protease concentrations and five substrate concentrations). The number of condition simultaneously assessed can easily go beyond one hundred, depending on the total number of droplets and the number of droplet housing similar conditions. After data collection and treatment, the effect of substrate and protease effect was determined (Figure 3&4). As expected, the fluorescent signal increases with time, with substrate concentration and with enzyme concentration.

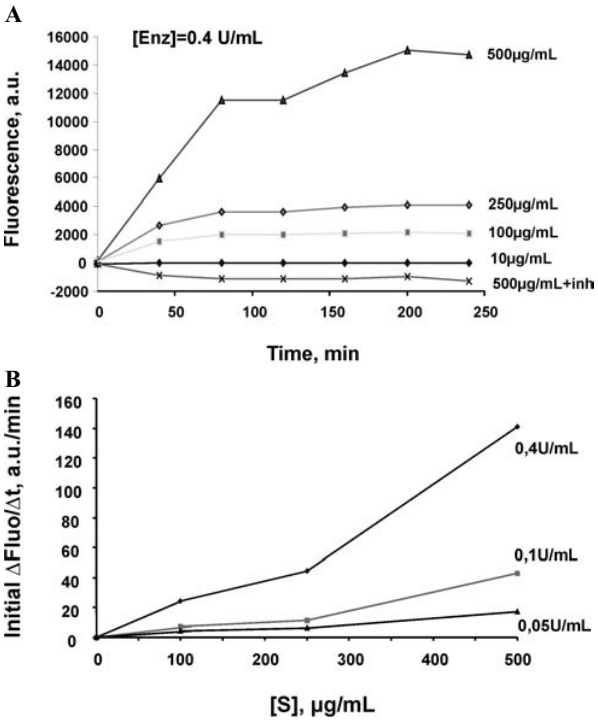

Figure 3: Effect of the substrate concentration on the rate of enzymatic reaction. (A.) The higher the substrate concentration (10 to 500 µg/mL), the brighter the fluorescent signal. Inhibitor signal remains under no-protease control. (B) Initial speed of fluorescent signal growth is roughly proportional to substrate concentration

Among assessed conditions we found out that for an enzyme concentration of 0.4 unit/mL, a proper substrate concentration should be around 100 µg/mL (Figure 3). We also observed that, with a substrate concentration of 250 µg/mL, an enzyme concentration of 0.02 unit/mL is suitable to monitor proteolytic activity (Figure 4). We finally concluded that a protease concentration of 0.05 unit/mL together with a substrate concentration of 250 mg/mL

would constitute a safe compromise to obtain a strong enough signal with minimal reagents quantities.

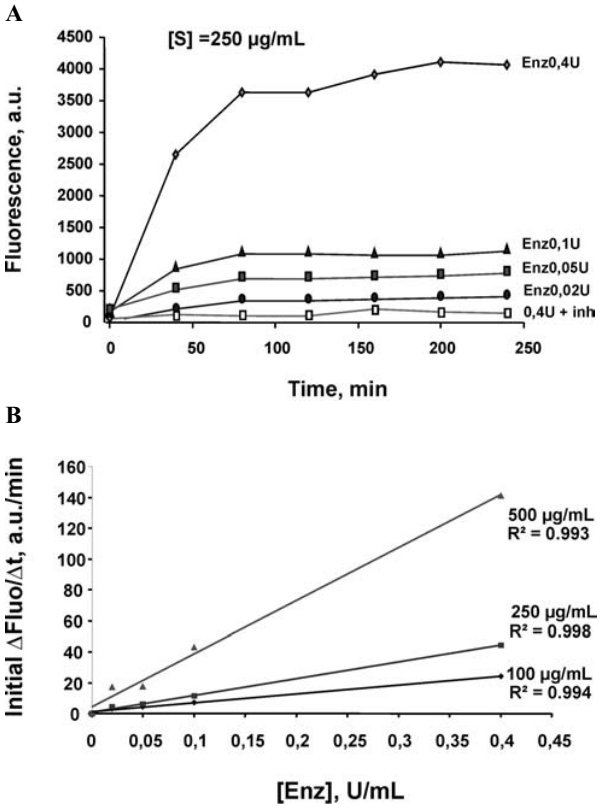

Figure 4: Effect of the protease concentration on the rate of enzymatic reaction. (A.) The fluorescent signal increases with protease concentration (0.01 to 0.4 U/mL), and remains close to zero in the presence of inhibitor. Inhibitor signal remains under no-protease control. (B) Initial speed of fluorescent signal growth is proportional to protease concentration.

3. CONCLUSION

The described tool is very efficient for monitoring hundreds of proteolysis micro-reactions simultaneously. One has the possibility to conveniently create in each droplet (each groups of droplet if working with duplicate or triplicate) a particular condition of reaction (reagents concentrations, buffer composition, inhibitors…). We successfully applied this versatile tool to reaction optimization and demonstrated inhibitor effect was unambiguously detectable within a wide range of enzyme concentration.

4. ACKNOWLEDGMENT

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